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Interdependence of H^+ and K^+ Fluxes During the Ca^{2+}-Pumping Activity of Sarcoplasmic Reticulum Vesicles

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The release of H^+ during the oxalate-supported Ca^{2+} uptake in sarcoplasmic reticulum vesicles is kinetically coincident with the initial phase of Ca^{2+} accumulation. The Ca^{2+} uptake is increased and the H^+ release is decreased in the presence of KCl and other monovalent chloride salts as expected for a H^+–monovalent cation exchange. The functioning of the Ca^{2+}-pump is disturbed by the presence of potassium gluconate and, to a lesser extent, of choline chloride. These salts do not inhibit the ATPase activity of Ca^{2+}-permeable vesicles, suggesting a charge imbalance inhibition which is specially relevant in the case of gluconate. Therefore, K^+, and also Cl^-, appear to be involved in secondary fluxes during the active accumulation of Ca^{2+}. The microsomal preparation seems homogeneous with respect to the K^+-channel, showing an apparent rate constant for K^+ release of approximately $25\,s^{-1}$ measured with the aid of $^{86}Rb^+$ tracer under equilibrium conditions. A Rb^+ efflux, sensitive to Ca^{2+}-ionophore, can be also detected during the active accumulation of Ca^{2+}. The experimental data suggest that both monovalent cations and anions are involved in a charge compensation during the Ca^{2+} uptake and H^+ release. Fluxes of these highly permeable ions would contribute to cancel the formation of a resting membrane potential through the sarcoplasmic reticulum membrane.

KEY WORDS: H^+ flux; K^+ flux; Ca^{2+}-pump; sarcoplasmic reticulum; rabbit muscle.

INTRODUCTION

Ca^{2+} uptake and release through the sarcoplasmic reticulum (SR)[2] network is the physiological trigger leading respectively to the relaxation and contraction of the muscle fiber (Ebashi and Endo, 1968). The movement of Ca^{2+} is accomplished through specific routes (Ca^{2+}-uptake pump and Ca^{2+}-release channel) located on the intracellular membrane (for reviews see Fleischer and Inui, 1989; McLennan, 1990; Inesi *et al.*, 1992). Beyond that, specific pathways allowing the rapid permeation of monovalent ions including H^+ (Meissner and Young, 1980), K^+, Na^+ (Meissner and McKinley, 1976; McKinley and Meissner, 1978; Ide *et al.*, 1991), or Cl^- (Meissner and McKinley, 1976; Tanifuji *et al.*, 1987; Rousseau *et al.*, 1988) have raised the question of the functional role played by these channels.

Studies on passive permeability of the SR membrane indicate that the mechanism of H^+ transport is different from that of K^+ and a H^+-K^+ exchange has been observed after the formation of opposing gradients of both cations (Meissner and Young, 1980).

Fluxes of monovalent ions have also been reported during the Ca^{2+}-pumping activity. Thus, a

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[2] Abbreviations used: SR, sarcoplasmic reticulum; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; FCCP, carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone.

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H^+ ejection was initially found during the inward Ca^{2+} transport (Madeira, 1978). This H^+ release was recognized to be dependent on the Ca^{2+} uptake and therefore sensitive to Ca^{2+}-ionophore but not to H^+-ionophore (Chiesi and Inesi, 1980).

Other evidence of the relationship between Ca^{2+} and monovalent cations come from indirect measurements. Transient membrane potentials (negative inside) have been shown to accelerate the rate of the ATP-dependent Ca^{2+} uptake and, vice versa, the Ca^{2+} transport process generates a membrane potential (positive inside) stimulating the release of H^+ and K^+ through specific channels (Meissner, 1981).

More recent studies, using a reconstituted system, have demonstrated the countertransport of Ca^{2+} and H^+ (Levy *et al.*, 1990) and the formation of a transmembrane electrical potential (Yu *et al.*, 1993) during the normal operation of the Ca^{2+}-pump.

A detailed description of the Ca^{2+} transport process at the molecular level requires the complete characterization of all the species that are co- or countertransported with Ca^{2+}. On the basis of previous findings and considering the Ca^{2+}-transporting properties of the ATPase protein in proteoliposomes we studied the H^+ and K^+ fluxes in native SR vesicles. An estimation of the H^+ and Ca^{2+} fluxes in the presence of different monovalent salts suggests that the interdependence of the H^+ and K^+ fluxes is secondary to the Ca^{2+} gradient. Additional experiments on $^{86}Rb^+$ permeability and $^{86}Rb^+$ efflux coupled to ATP-dependent $^{45}Ca^{2+}$ influx confirm the relationship of the K^+ and Ca^{2+} fluxes. All these data can be integrated in a compensating mechanism to neutralize the electrochemical Ca^{2+} gradient brought about by the functioning of the Ca^{2+}-pump.

MATERIALS AND METHODS

Preparation of SR Vesicles

Sarcoplasmic reticulum vesicles were isolated from white skeletal muscle of New Zealand rabbit, according to the method of Eletr and Inesi (1972). Protein concentration was determined by the method of Lowry *et al.* (1951) using bovine serum albumin as standard.

H^+ Flux

Light transmission change of chlorphenol red was used to monitor H^+ fluxes during the Ca^{2+}-pumping activity (Chiesi and Inesi, 1980). The experimental signal was measured at 25°C and pH 6.0 under continuous stirring by dual-wavelength spectrophotometry using a modular optical system from Bio-Logic Co. (Claix, France). Samples were excited with white light and analyzed at 480.4 and 560.7 nm by two photomultipliers. The wavelength selection was made by placing narrow-bandwidth interference filters (Ealing Electro-Optics, Holliston, Massachusetts) in front of each photomultiplier. The microsomal vesicles were preincubated for 10 min before any measurement. The composition of each reaction mixture is described in the corresponding figure legend. H^+ production was also measured at pH 6.0 in the presence of 0.1% Triton X-100 to evaluate the amount of H^+ as a consequence of the hydrolysis of the phosphorylating substrate. This component was subtracted from the H^+ generated by intact vesicles to determine the fraction of H^+ release. Changes in the recorded signal upon addition of 10 μM HCl aliquots were used for calibration.

Movement of Ca^{2+}

Ca^{2+} flux through the SR membrane was monitored by spectrophotometry in a dual-wavelength mode as described for the H^+ flux. The selected wavelength pair was 660.4 and 680.1 nm using the metallochromic indicator Arsenazo III. A standard Ca^{2+} solution was used for calibration at the end of each experiment. Ca^{2+} transport was also measured by $^{45}Ca^{2+}$ radioactive tracer. The reaction mixture contained 2 mM Mes (pH 6.0), 5 mM $MgCl_2$, 50 μM $^{45}CaCl_2$ ($\sim$ 5,000 cpm/nmol), 0.1 mg protein/ml, and different concentrations of salts as described in the figure legend. The reaction was initiated by adding 1 mM acetylphosphate and stopped at different time intervals by filtering 1 ml aliquots through HAWP Millipore filter (0.45 μm). The filters were rinsed with 2 ml of medium 2 mM Mes (pH 6.0), 5 mM $MgCl_2$, and 1 mM EGTA; the Ca^{2+} transport rates were calculated from the initial phase of the experimental time points.

Ca^{2+}-ATPase Activity

The hydrolytic activity of leaky vesicles was assayed at 25°C in a medium containing 2 mM Mes (pH 6.0), 5 mM $MgCl_2$, 50 μM $CaCl_2$, 10 μM A23187, 0.1 mg protein/ml, 1 mM ATP, and different concentrations of monovalent salt. P_i production over the

first minutes of the reaction was evaluated from the linear slope by a colorimetric method (Lin and Morales, 1977). Basal ATPase activity (Mg^{2+}-dependent) measured in the presence of 1 mM EGTA was subtracted in each case.

Isotopic Exchange of Rb^+

SR vesicles (1.5 mg protein/ml) were equilibrated for 30 min at room temperature in a medium consisting of 2 mM Hepes (pH 7.0), 1 mM glucose, and 1 mM $^{86}RbCl$ (~ 5,000 cpm/nmol). Thereafter, a trace amount of [^{3}H]-glucose (~ 5,000 cpm/nmol) was added to the incubation mixture and aliquots of 0.2 ml (0.3 mg protein) were placed onto HAWP Millipore filters (0.45 μm) under vacuum. Filters were rapidly flushed at selected times with unlabeled medium of identical composition by using a rapid filtration device (Dupont and Moutin, 1987). The amount of ^{3}H retained by the filters allowed determining the $^{86}Rb^+$ remaining inside the vesicles (Yamaguchi and Watanabe, 1988). In some experiments, the RbCl concentration and/or the pH of the incubation and flushing media were changed as specified.

Rb^+ Release during the Ca^{2+} Uptake

The Rb^+ content inside the vesicles was determined by a double-labeling filtration technique as described before (Orlowski and Champeil, 1991). The microsomal vesicles (0.5 mg protein/ml) were incubated for 30 min at room temperature in the presence of 2 mM Mes (pH 6.0), 5 mM $MgCl_2$, 1 mM $^{86}RbCl$ (~ 5,000 cpm/nmol), 1 mM glucose, and 90 μM $CaCl_2$. Carrier-free [^{3}H]-glucose (~ 5000 cpm/nmol) was added to the incubation medium, and aliquots of 0.8 ml (0.4 mg) were filtered (HAWP Millipore filter) by hand to evaluate the Rb^+ retained by the vesicles. The release of Rb^+ during the Ca^{2+}-translocating activity was measured at various times after addition of 1 mM ATP by the same procedure. The Ca^{2+} accumulated by the vesicles was determined in parallel experiments by including $^{45}CaCl_2$ (~ 2,000 cpm/nmol) and omitting the radioactive labels $^{86}Rb^+$ and [^{3}H]-glucose in the incubation medium. In this case, the nonspecific free Ca^{2+} trapped in each filter was eliminated by rinsing the filters with 2 ml of medium 2 mM Mes (pH 6.0), 1 mM RbCl, and 1 mM EGTA. 10 μM A23187 was included in the control experiments.

Miscellaneous

Arsenazo III, sodium salt (approx. 98%) was purchased from Sigma Quimica (Spain). The radioactive tracers $^{86}Rb^+$ and $^{45}Ca^{2+}$ were obtained as chloride salts from Amersham Iberica (Spain), and [^{3}H]-glucose was from New England Nuclear (Germany). Ionophore A23187 from Boehringer Mannheim (Germany) was prepared as ethanolic solution. Choline chloride stock solution was aliquotted and frozen until use to avoid spontaneous decomposition. Mes and Hepes buffers were titrated at pH 6.0 and 7.0, respectively, by addition of Tris. The spectrophotometric traces shown in the figures correspond to representative experiments.

RESULTS

The functional activity of the SR membrane involves different types of ionic fluxes associated with the Ca^{2+} transport. We initially selected experimental conditions to measure a H^+ flux associated with the ATPase-dependent Ca^{2+} entry. When the SR vesicles are incubated in a slightly buffered medium at pH 6.0 containing 5 mM $MgCl_2$, 60 μM $CaCl_2$, 25 μM Arsenazo III, 2 mM potassium oxalate, and 1 mM acetylphosphate, a biphasic profile of Ca^{2+} uptake (Madeira, 1984) can be observed. This profile was specially well defined and slow (dotted line in Fig. 1A) due to the nature of the energy-donor substrate, the metallochromic indicator used, and the absence of KCl. The formation of a H^+ gradient can be demonstrated by recording changes in the optical signal of the H^+-indicator chlorphenol red. The experimental trace (solid line in Fig. 1A) indicates that the appearance of the H^+ gradient is kinetically coincident with the initial phase of the Ca^{2+} transport process.

These preliminary experiments were then repeated in the presence of 80 mM KCl. As can be seen in Fig. 1B, KCl accelerates the rate of the Ca^{2+} uptake (dotted line) and decreases the rate of H^+ release (solid line). Furthermore, both ionic gradients develop simultaneously as before, and the steady level of the H^+ gradient formed was considerably lower than that observed in the absence of KCl.

The effect of K^+ on the H^+ efflux was studied in the absence of oxalate building up A23187-sensitive H^+ gradients (see Discussion). The time dependence of H^+ efflux indicates that the magnitude of the ΔpH

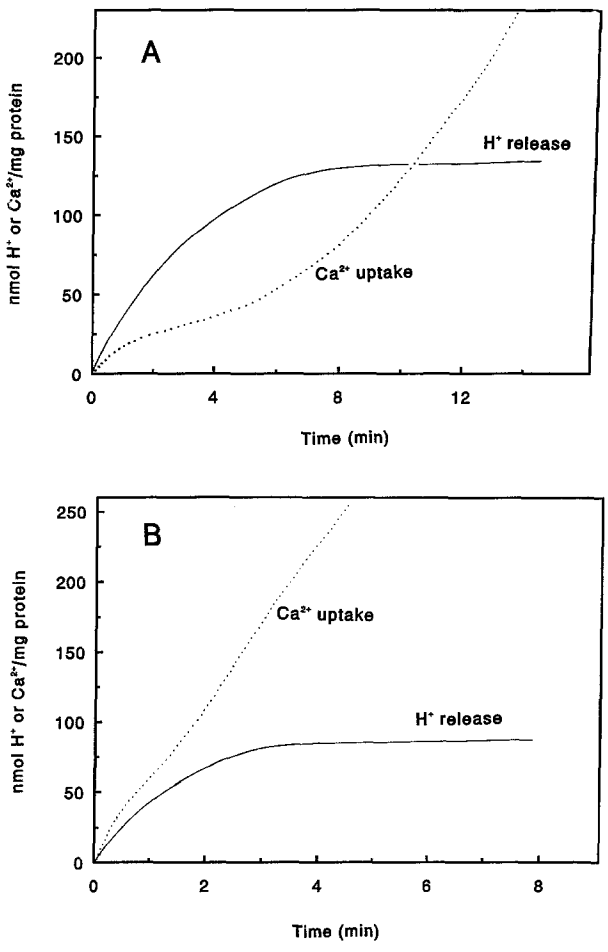

Fig. 1. Direct recording of H^+ release during the active accumulation of Ca^{2+} in the presence of oxalate. The experiments were carried out by the optical method at 25°C in a medium containing 2 mM Mes (pH 6.0), 5 mM $MgCl_2$, 60 μM $CaCl_2$, 2 mM potassium oxalate, and 0.1 mg SR protein/ml. The reaction was started by adding 1 mM acetylphosphate. The H^+ fluxes were measured in the presence of 40 μM chlorphenol red and the wavelength selection was 480.4 and 560.7 nm. To measure the Ca^{2+} uptake, we used 25 μM Arsenazo III, and the dual wavelength was 660.4 and 680.1 nm. The KCl concentration was zero (panel A) or 80 mM (panel B).

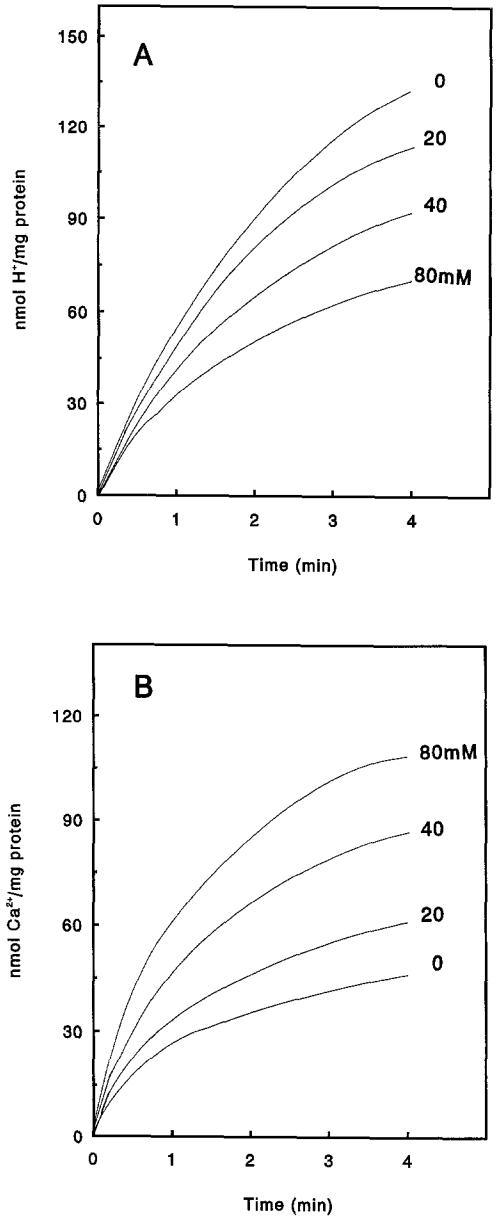

Fig. 2. Effect of KCl on H^+ efflux (A) and Ca^{2+} influx (B) in Ca^{2+}-translocating SR vesicles. The experimental conditions are essentially as described for Fig. 1 but in the absence of oxalate. The protein concentration was 0.3 mg/ml and the KCl concentrations (mM) are indicated on each trace. In A, the H^+ efflux curves have been obtained by difference between the H^+ production in intact and leaky (0.1% Triton X-100) SR vesicles at each KCl concentration.

formed and the apparent initial rate of the process are influenced, in a concentration-dependent manner, by the KCl concentration present. As the KCl concentration increases from 0 to 80 mM, the apparent H^+ transport is progressively diminished (Fig. 2A). By contrast, the Ca^{2+} transport profiles (Fig. 2B) indicate that the rate of Ca^{2+} translocation and the intravesicular Ca^{2+} gradient increase as a function of the KCl concentration.

The effect of monovalent cations other than K^+ can be studied by including different concentrations of NaCl, NH_4Cl, or LiCl in the incubation medium. The experiments were performed as indicated above for K^+, and the initial rates of H^+ and Ca^{2+} fluxes, expressed as nmol/min and mg protein, were plotted as a function of the corresponding monovalent salt concentration. Thus, the rate of the net H^+ efflux that can be monitored by the pH-indicating dye was reduced by the presence of the chloride salts although

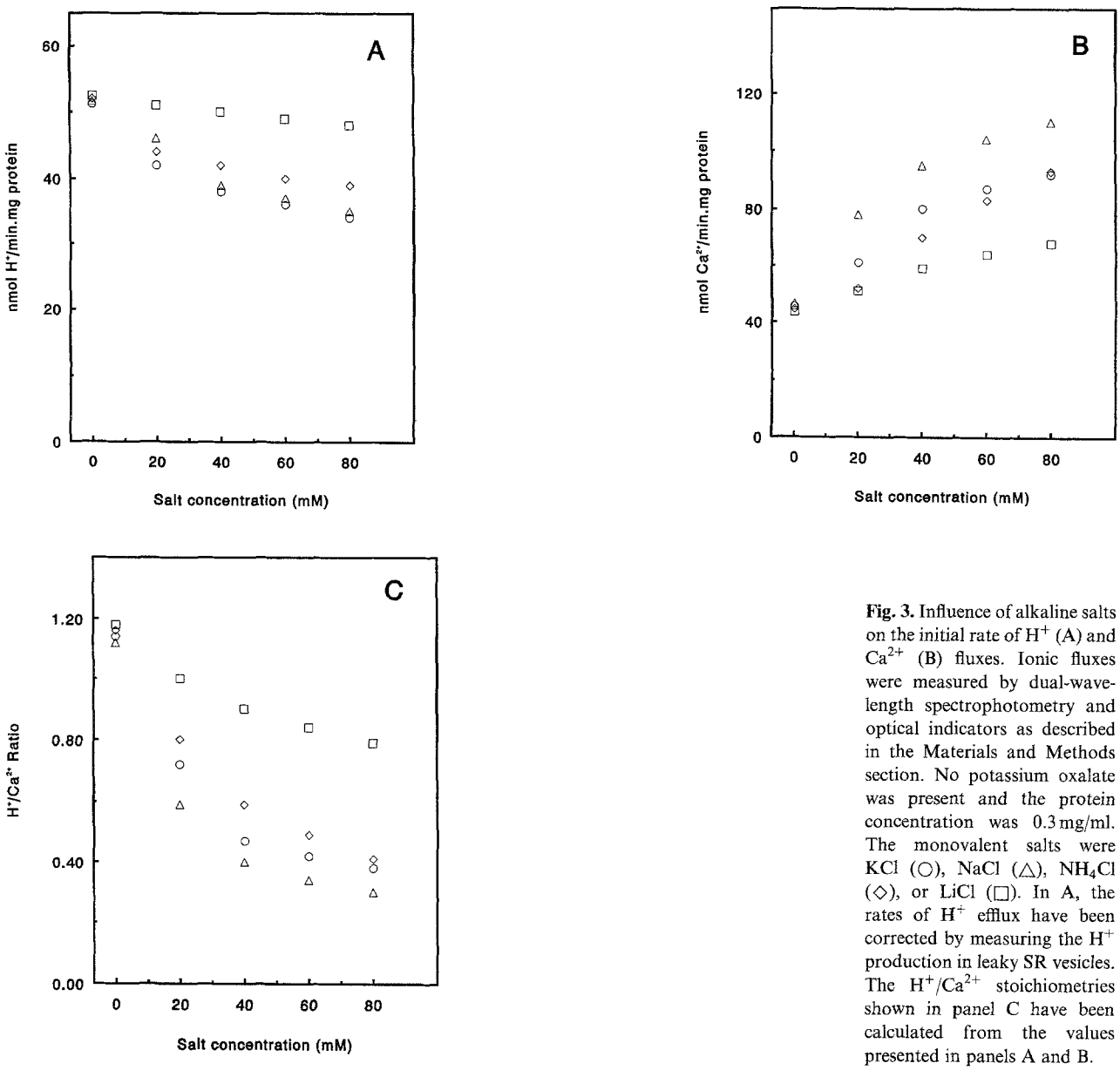

Fig. 3. Influence of alkaline salts on the initial rate of H^+ (A) and Ca^{2+} (B) fluxes. Ionic fluxes were measured by dual-wavelength spectrophotometry and optical indicators as described in the Materials and Methods section. No potassium oxalate was present and the protein concentration was 0.3 mg/ml. The monovalent salts were KCl (○), NaCl (△), NH_4Cl (◇), or LiCl (□). In A, the rates of H^+ efflux have been corrected by measuring the H^+ production in leaky SR vesicles. The H^+/Ca^{2+} stoichiometries shown in panel C have been calculated from the values presented in panels A and B.

the Li^+ salt appears to be the least effective (Fig. 3A). Conversely, the rates of the Ca^{2+} transport measured with the metallochromic indicator are stimulated by similar concentrations of the referred monovalent salts (Fig. 3B). In this case, Li^+ is the least active cation in promoting the activation of the Ca^{2+}-pump.

The apparent effect of monovalent cations on the H^+ flux can be more properly analyzed by considering the H^+/Ca^{2+} ratio. This allows one to correct for the differential effect of the cations on the Ca^{2+}-pump activity. Figure 3C shows that all of them display a general trend to decrease the H^+/Ca^{2+} ratio as the concentration of salt increases. It is apparent that K^+, Na^+, and also NH_4^+ show a similar behavior, whereas Li^+ appears to be less effective to relieve the H^+ gradient (presents higher H^+/Ca^{2+} ratios).

We can also dissect separately the effect of K^+ and Cl^- as charge compensation ions. This was accomplished by using potassium gluconate salt, thus substituting chloride by a relatively impermeant anion. In the other case, potassium was replaced by the less permeant cation choline. In this way we can assume that the relatively low permeability of gluconate$^-$ and choline$^+$ will preclude the movement of these ions across the membrane during the time course of our

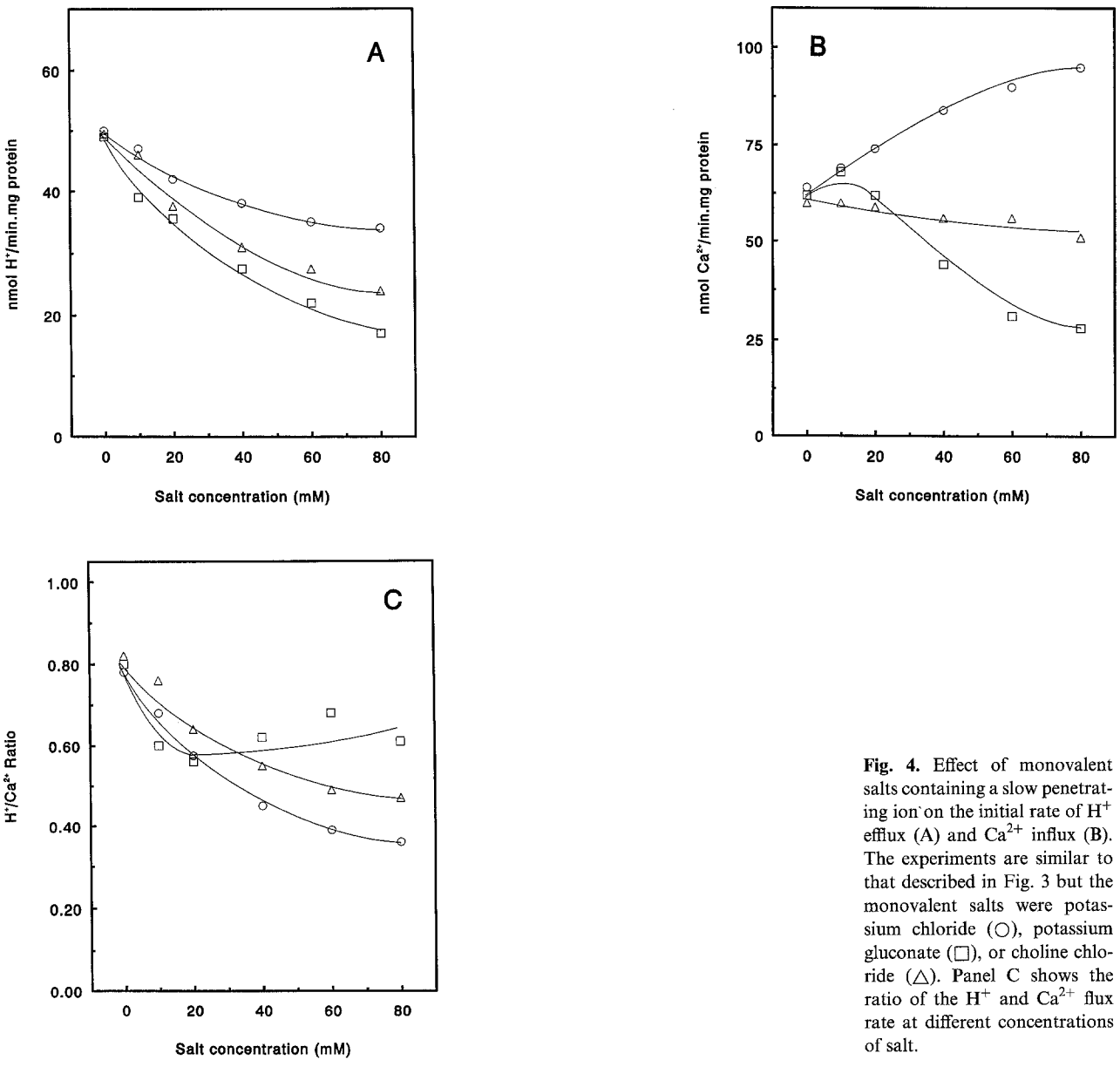

Fig. 4. Effect of monovalent salts containing a slow penetrating ion on the initial rate of H^+ efflux (A) and Ca^{2+} influx (B). The experiments are similar to that described in Fig. 3 but the monovalent salts were potassium chloride (○), potassium gluconate (□), or choline chloride (△). Panel C shows the ratio of the H^+ and Ca^{2+} flux rate at different concentrations of salt.

ion flux measurements. The results of Fig. 4A indicate that potassium gluconate or choline chloride are able to decrease the rate of the net H^+ efflux. This effect is a function of the salt concentration as in the case of KCl.

The initial rates of the inward Ca^{2+} movement measured under similar conditions indicate that the rate of Ca^{2+} accumulation is slightly inhibited by choline chloride up to a concentration of 80 mM. Potassium gluconate exerts a clear inhibitory effect specially at the higher concentrations tested (Fig. 4B). The activating effect of KCl on the Ca^{2+} uptake rate was also shown as a control.

To better understand the origin of the decrease in H^+ efflux, we should refer again to the ratio of H^+ and Ca^{2+} fluxes. Thus, Fig. 4C reveals that choline chloride behaves like KCl, decreasing the Ca^{2+}-dependent H^+ gradient. However, the effect of potassium gluconate was quite different since the decrease in H^+ flux was accompanied by a drastic effect on Ca^{2+} transport. Therefore, the H^+/Ca^{2+} ratio undergoes an initial decrease followed by an increase which is specially evident at the higher concentrations of salt.

Experiments on Ca^{2+}-ATPase activity yielded an additional clue to interpret the preceding results. We followed the ATP hydrolytic activity of A23187-

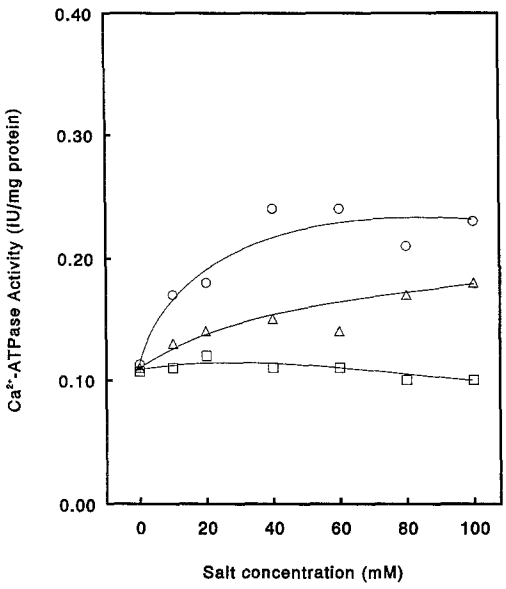

Fig. 5. Rates of the Ca^{2+}-ATPase activity in the presence of different monovalent salts. The enzymatic assay was performed at 25°C by the molybdovanadate method (Lin and Morales, 1977). The reaction medium was 2 mM Mes (pH 6.0), 5 mM $MgCl_2$, 50 μM $CaCl_2$, 10 μM A23187, 0.1 mg SR protein/ml, 1 mM ATP, and different concentrations of KCl (○), potassium gluconate (□), or choline chloride (△). The Ca^{2+}-dependent ATPase activity was obtained by subtracting the basal ATPase from the total activity.

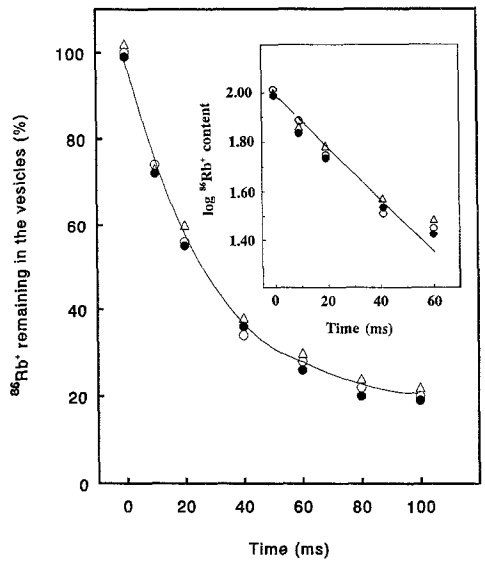

Fig. 6. Kinetics of passive Rb^+ release under conditions of equilibrium. SR vesicles were equilibrated for 30 min at room temperature in different incubation media containing 1 mM glucose, $^{86}RbCl$ (~ 5000 cpm/nmol), and 1.5 mg protein/ml. Other components are specified below. A trace amount of [^{3}H]glucose was added before starting the experiments, and 0.2 ml aliquots were used to follow the process at sequential time intervals. For each experimental point, the sample suspension (0.3 mg protein) was added onto Millipore filter (HAWP) placed on a rapid filtration apparatus. The exchange was triggered by perfusing the filter content in the millisecond time scale with the corresponding nonlabeled incubation medium. Counting of $^{86}Rb^+$ and $^3H^+$ in the suspension and the filters was used to calculate the Rb^+ (nmol/mg protein) inside the vesicles. Other components of the incubation media: 2 mM Mes (pH 6.0), 1 mM RbCl (●); 2 mM Hepes (pH 7.0), 0.1 mM RbCl (△); or 2 mM Hepes (pH 7.0), 1 mM RbCl (○). The inset shows a first-order kinetics plot of the experimental data.

treated vesicles to assess whether the monovalent salts have a direct effect on the enzyme turnover. The results shown in Fig. 5 indicate that the salts under study do not inhibit the hydrolytic activity of the Ca^{2+}-pump. In fact, KCl and choline chloride accelerate the Ca^{2+}-ATPase activity of Ca^{2+}- permeable vesicles when used in the millimolar concentration range, the potassium salt being more effective than that of choline. On the other hand, the presence of potassium gluconate did not practically alter the rate of ATP hydrolysis when compared with the control experiment in the absence of salt.

A direct characterization of the K^+ permeability can be obtained by using $^{86}Rb^+$ as a surrogate tracer for K^+ and a rapid kinetics technique. In these experiments, SR vesicles were subjected to incubation with radioactive $^{86}Rb^+$ to reach the chemical equilibration. Then, the isotopic exchange was elicited by flushing the samples with unlabeled RbCl. The experiment requires a rapid filtration apparatus to follow the exchange reaction in the millisecond time scale and [^{3}H]-labeled glucose as a second tracer to correct for extravesicular $^{86}Rb^+$ retained by the filters. This is an absolute requirement to get reliable values at the shorter time points owing to the very high permeability of this membrane for Rb^+. In Fig. 6 we show the time resolution of the $^{86}Rb^+$ outflow expressed as percentage of the tracer retained after perfusion. The rate of exchange was independent of the initial RbCl concentration used (0.1 or 1 mM), and no effect of the reaction medium pH (6.0 or 7.0) was detected. From the slope of the semilog plot (inset of Fig. 6) we can estimate an apparent rate constant for the equilibrium exchange of approximately 25 s^{-1}.

We were also able to detect the transmembrane K^+ movement associated to the active accumulation of Ca^{2+} with the aid of $^{86}Rb^+$. The experiments were started after 30 min incubation in a medium to measure Ca^{2+} uptake supplemented with $^{86}Rb^+$. The initial time points of Fig. 7 reflect the level of the radioactive Rb^+ accumulated at equilibrium. Upon

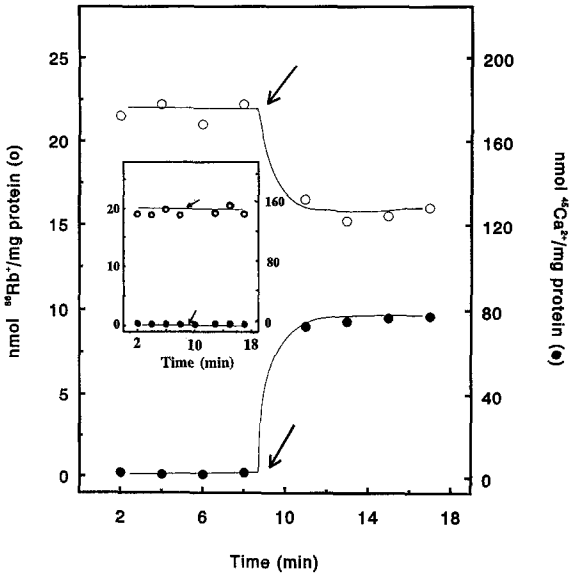

Fig. 7. Dependence of the Rb^+ efflux on the inward Ca^{2+} movement in SR vesicles. The reaction medium contained 2 mM Mes (pH 6.0), 5 mM $MgCl_2$, 1 mM $^{86}RbCl$, 1 mM glucose, 90 μM $CaCl_2$, and SR vesicles (0.5 mg protein/ml). After 30 min incubation at room temperature to reach the chemical equilibration, [^{3}H]glucose (~ 5000 cpm/nmol) was added. Then, aliquots of 0.8 ml were withdrawn and filtered (0.45 μm pore size) by hand at different times before and after addition of 1 mM ATP. The double labeling was used to determine the Rb^+ retained inside the vesicles. Similar experiments were carried out in the presence of $^{45}CaCl_2$ (~ 2000 cpm/nmol) but containing nonradioactive Rb^+ and glucose. The Ca^{2+} content inside the vesicles was measured after filtration and rinsing of the filters with 2 ml of medium 2 mM Mes (pH 6.0), 1 mM RbCl, and 1 mM EGTA. The same experiments were performed by including 10 μM A23187 in the reaction mixture (inset). Labels of the axes and symbols in the inset are the same as in the main frame. Timing of the ATP additions is marked by arrows.

addition of ATP the content of the radioactive tracer was measured again. It is observed that the addition of ATP resulted in the release of Rb^+ to reach a lower level of accumulation. Moreover, it is proved that the release of Rb^+ is accompanied by the ATP-dependent Ca^{2+} entry inside the vesicles. This fact confirms the relationship of the K^+ and Ca^{2+} fluxes. Furthermore, control experiments (inset of Fig. 7) indicated that no ionic fluxes can be detected when the Ca^{2+}-ionophore A23187 was included in the reaction medium.

DISCUSSION

The study of ionic fluxes in SR vesicles can benefit from the knowledge derived of the Ca^{2+}-ATPase in reconstituted systems with no electrolyte leak. Our goal in the present study was directed to understanding the dynamic interplay among the Ca^{2+}, H^+, and K^+ fluxes. The biphasic profile of the Ca^{2+} uptake that we measured in the presence of Arsenazo III is parallel to that observed by using a Ca^{2+} electrode (Madeira, 1984). Nevertheless, the potential effect of this dye on the Ca^{2+}-pump activity should be stressed (Riollet and Champeil, 1987).

Since it is known that a highly permeant K^+ channel exists in this membrane (Meissner and McKinley, 1976; McKinley and Meissner, 1978), we initially removed KCl from the reaction medium to simplify the ion fluxes under consideration. In the initial experiments performed in the presence of acetylphosphate and oxalate we could observe a H^+ efflux coupled to the active accumulation of Ca^{2+}. This H^+ movement was strictly limited to the initial phase of the Ca^{2+} transport. Thereafter, a massive accumulation of this cation takes place due to the low solubility product of the oxalate salt.

We noted that ionophore A23187 does not apparently collapse the H^+ gradient formed in the presence of oxalate as it does in its absence (data not shown), suggesting that the H^+ flux observed under these conditions could be related to a H^+ displacement from oxalate. This prompted us to use an oxalate-free medium in our experiments.

Some studies (Meissner, 1981), making use of H^+ or K^+-induced membrane potentials and fluorescent dyes, conclude that the active transport of Ca^{2+} stimulates the release of H^+ and K^+. The present data obtained in functional Ca^{2+}-pumping vesicles also indicate the release of H^+ and K^+ and are in favor of the interdependence of the H^+ and K^+ fluxes. Our experimental approach avoids the osmotic effects that can be created (Meissner and McKinley, 1976) during the loading and dilution procedures to impose artificial membrane potentials. It also facilitates the kinetic study of the H^+ flux by slowing down the rate of the Ca^{2+} internalization.

Some indirect information can be withdrawn by considering the effect of the alkali metal cations. The permeability of the SR membrane for these cations is extremely high (Meissner and McKinley, 1976) when compared with the permeation rate of Ca^{2+} during the uptake. Therefore, if a direct-coupling Ca^{2+}-monovalent cation exists, it is not expected to see any difference in the rate of this exchange for the different cations. The observed differential effect suggests that the movement of these cations becomes rate-limiting. This can be expected for a monovalent

cation–H^+ exchange under conditions of a slow-forming H^+ gradient as in our case. An additional argument in favor of the Ca^{2+}–H^+ exchange and against that of Ca^{2+}–monovalent cation is the strong increase in the Ca^{2+} pumping activity induced by FCCP in reconstituted systems with respect to that observed in the presence of valinomycin (Levy *et al.*, 1990; Yu *et al.*, 1993).

Comparison of the H^+/Ca^{2+} ratio in the presence of monovalent chloride salts give us an estimation of the extent of the monovalent cation–H^+ exchange. The efficiency of this exchange, $K^+ \simeq Na^+ > NH_4^+ > Li^+$, is compatible with the relative permeability of these cations through the SR membrane (Meissner and McKinley, 1976).

The stoichiometry of the H^+/Ca^{2+} fluxes in native SR vesicles varies as a function of the monovalent salt concentration. Higher values were obtained in the absence of salt and conditions of maximal Ca^{2+} uptake. When measured from the initial rate of the fluxes (see Figs. 3C and 4C), the value is in the range of 0.8 to 1.2, being in agreement with the H^+/Ca^{2+} ratios reported for reconstituted Ca^{2+}-ATPase vesicles (Levy *et al.*, 1990; Yu *et al.*, 1993). Values considerably higher than 1 can be obtained from the steady level (Fig. 2A and B). If this is the case, an additional component of H^+ release could be the result of H^+ displacement after binding of Ca^{2+} to low-affinity sites inside the vesicles. In any case the absolute value of this ratio is very difficult to assess owing to the leakiness of the SR membrane.

The Ca^{2+} entry inside the vesicles is an electrogenic process as deduced from reconstitution experiments (Yu *et al.*, 1993). Therefore, transient fluxes of electrolytes can be expected in the native vesicles to offset the net charge displacement. It is known that the permeation rate of K^+ or Cl^- is about 10 times faster than that of gluconate or choline ions (Meissner and McKinley, 1976). Accordingly, we used reaction media containing a fast (K^+ or Cl^-) and a slow (gluconate$^-$ or choline$^+$) penetrating counter-ion. The Ca^{2+} fluxes were measured by the radiometric procedure in order to avoid feasible optical interferences although similar results were obtained with the metallochromic indicator. The analysis of flux data in conjunction with that of ATPase activity can be summarized as follows. Choline chloride diminishes the H^+ gradient and, to some extent, the Ca^{2+} uptake capacity of native vesicles while activating the Ca^{2+}-ATPase activity of leaky vesicles. Potassium gluconate decreases the H^+ efflux rate in association with a marked decrease in the Ca^{2+} influx and no alteration of the ATPase activity. Therefore, the inhibition of the Ca^{2+} fluxes may be attributed to the formation of a membrane potential rather than to a direct effect on the molecular mechanism of the Ca^{2+}-pump. This is specially important in the case of potassium gluconate where the fixed negative charges in the external medium will severely restrain the transport of Ca^{2+} (positive charges). In this regard, the formation of a membrane potential has been reported to interfere with the Ca^{2+} uptake capacity in the ATPase-containing proteoliposomes.

The above results suggest that K^+ may be partially substituted by Cl^- to neutralize the electric charge generated during the Ca^{2+} transport process. However, the transmembrane movement of Cl^- in the same direction as Ca^{2+} would create osmotic effects. Therefore, the counter-movement of K^+ seems more favorable under physiological conditions. Besides, K^+ is the prevalent ion in the SR compartment and in the surrounding medium (Somlyo *et al.*, 1977).

Available data on the ionic permeability of the SR membrane come from indirect methods such as change in the vesicle volume monitored by light-scattering (Kometani and Kasai, 1978) or fluorescence quenching of Tl^+ measured by stopped-flow technique (Garcia and Miller, 1984). Radioactive tracer fluxes have also been measured by the conventional filtration technique although the very fast permeation rate of K^+ cannot be adequately followed (McKinley and Meissner, 1978). Here, we have combined this direct method and a time-resolved technique to obtain a more precise kinetic characterization of the K^+-channel. The isotope exchange experiments at equilibrium show that Rb^+ leaves the vesicles following an apparent first-order rate constant, which is an indication of a K^+ single pool. From the first-order Rb^+ release with an apparent rate constant of $\sim 25\,s^{-1}$ and a vesicle diameter of 0.1 μm (Meissner, 1975), we can estimate a permeability coefficient for Rb^+ on the order of 10^{-5} cm/s. Likewise, an initial Rb^+ efflux rate of 572 nmol/s and mg protein can be calculated assuming a Rb^+ concentration of 22 nmol/mg protein. These values are considerably higher than those previously reported (McKinley and Meissner, 1978).

The initial rate of the Ca^{2+} uptake in SR vesicles is 60–70 nmol/s per mg protein (Inesi and Scarpa, 1972);

therefore, Rb^+ and hence K^+ can cross the membrane with a rate that is roughly one order of magnitude greater than that of Ca^{2+}-pumping, suggesting that no significant membrane potential changes may be produced during muscle relaxation.

Addition of typical K^+-channel blockers such as tetraethylammonium or 4-aminopyridine failed to stop the Rb^+ exchange (not shown), suggesting a different nature for the K^+ pathway (channel) in the SR membrane.

Our observations support the idea that highly permeable ions will contribute to the compensation of the displacement of mass and charge induced by the Ca^{2+}-pump. These data can be compiled in a functional model comprising a primary active pump and a secondary equilibrating system. Assuming that the Ca^{2+}-pump is an obligatory $1:1\ Ca^{2+}/H^+$ exchanger, all our data can be explained as follows. The inwardly directed Ca^{2+} gradient would lead via the Ca^{2+}/H^+ exchange to the acidification of the extravesicular space and the formation of a membrane potential. The H^+ gradient and the net positive charge inside the vesicles will promote the K^+ efflux via independently coupled pathways of H^+-K^+ exchange. This should be considered as a basic model at present as fluxes of other ions including that of Cl^- cannot be discounted.

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